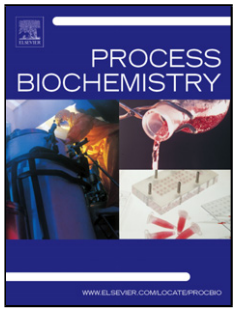


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Author: Zhi-Jian Tan Chao-Yun Wang Yong-Jian Yi Hong-Ying Wang Wan-Lai Zhou Shi-Yong Tan Fen-Fang Li

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## Highlights

- TPP was used for simultaneous purification of aloe polysaccharide and protein by a single-step extraction.
- Aloe polysaccharide was further purified by dialysis to remove the salt after TPP.
- SDS-PAGE was used to analyze aloe protein.
- The extraction efficiencies of aloe polysaccharide and protein were 92.26% and 92.78% respectively under the optimized conditions.

# Three phase partitioning for simultaneous purification of aloe polysaccharide and protein using a single-step extraction

Zhi-Jian Tan [1, 2], Chao-Yun Wang *[1], Yong-Jian Yi [1], Hong-Ying Wang [1], Wan-Lai Zhou [1], Shi-Yong Tan [1], Fen-Fang Li [2]

*(1. Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha 410205, China;*

*2. College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China)*

**ABSTRACT:**

In this paper, an inexpensive, rapid and efficient three phase partitioning (TPP) technique was used to simultaneously purify aloe polysaccharide (APS) and protein using a single-step extraction. This TPP system is established by adding ammonium sulfate and *t*-butanol to the crude slurry of aloe powder. APS was extracted in the lower phase, and the protein was extracted in the middle phase. The extraction conditions optimized were mass concentration of ammonium sulfate and *t*-butanol, temperature and pH. APS was further purified using a dialysis membrane to remove the salt, and its purity was determined. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze aloe protein. TPP is an attractive and potential technique for the purification of APS and protein from *Aloe vera* L.

**Keywords:** Three phase partitioning, aloe polysaccharide, aloe protein, purification

## 1. Introduction

*Corresponding author. Tel.: +86 731 88998501; fax: +86 731 88998501.
E-mail address: ibfcwcy@139.com (C. Y. Wang).

*Aloe vera* L. (*Aloe barbadensis* Miller) is a succulent and perennial plant belonging to the Liliaceae family [1, 2]. *Aloe vera* mainly grows in arid areas of Africa, Asia and North America and it is widely used in food, pharmaceutical and cosmetic industries [3]. *Aloe vera* contains 75 potentially active compounds, including polysaccharides, proteins, minerals, phenolic compounds, vitamins, amino acids, *etc.* [4, 5]. Aloe polysaccharide (APS) is the main active ingredient in aloe gel, which is responsible for the gel's wound healing, anti-inflammatory and immunomodulatory properties [6, 7]. Aloe protein is another important ingredient in aloe gel, but there are few studies on it. Siritapetawee [8] studied the inhibition of the fibrinogenolytic and fibrinolytic activities of plasmin by a protease inhibitor protein (molecular weight of 11.8 kDa) isolated from Aloe *vera* leaf gel, suggesting its potential use as an antifibrinolytic treatment. Das [9] reported on the anti-fungal and anti-inflammatory properties of a protein (molecular weight of 14 kDa) isolated from aloe gel. Many methods are reported for the extraction and purification of APS, such as alcohol precipitation [10], ion-exchange chromatography coupled with gel permeation chromatography [11-13], membrane separation [14], and aqueous two-phase extraction [15]. Although these methods seem to be effective, there are many disadvantages, including high cost and time demands, or difficulty in scale-up. Alcohol precipitation can be used as a pretreatment process, and further treatments are needed to obtain APS with high purity. Ion-exchange chromatography coupled with gel permeation chromatography for the purification of APS will require more cost and time. Membrane separation is an effective method for the purification of APS, but the

membrane is easily ruined by aloe gel with high viscosity. In our previous studies, an ionic liquid-based aqueous two-phase system (IL-ATPS) was used for simultaneous extraction and purification of APS and protein [15]. IL-ATPS is an effective method, but unlike TPP extraction, pretreatment via alcohol precipitation was needed to obtain the crude APS with protein being discarded as an impurity. Furthermore, IL was expensive, and protein extracted into the IL-rich phase should be separated from IL.

In recent years, TPP has emerged as an inexpensive, rapid and efficient technique for the separation and purification of enzymes, proteins [16-20], and edible oils [21, 22]. TPP is easily scalable and can be used directly with crude slurry, it is performed at room temperature, and it does not use polymers, which have to be removed later [23, 24]. TPP is often formed by adding water miscible aliphatic alcohol and salt to a slurry of protein, forming an alcohol-rich upper phase, solid middle phase of protein and salt-rich lower phase [25-27]. The pigments, lipids, and hydrophobic materials are concentrated in the upper phase, the protein and cell debris are concentrated in the middle phase, saccharides and other polar components are concentrated in the lower phase [28]. The principles of TPP are very complex and involve numerous techniques such as salting out, cosolvent, isoionic, and osmolytic and kosmotropic precipitation of protein [29, 30]. In this paper, TPP was used for the separation and purification of APS and protein via a single-step extraction. The complete flow chart is shown in Fig. S1.

## 2. Materials and methods

*2.1 Materials and reagents*

The dried aloe pulp powder was obtained from NanTong DeFu Aloe Products Co. Ltd. (NanTong, China). Mannose, bovine serum albumin (BSA), and Coomassie Brilliant Blue G-250 were acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ammonium sulfate and *t*-butanol were of analytical grade and acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

*2.2 Preparation of TPP system*

The crude slurry was prepared by dissolving the dried aloe pulp powder into water. This aloe powder was obtained by grinding the dried aloe leaf to a particle size of 60 mesh. The insoluble material of this aloe powder was approximately 32.18% (w/w), which was removed by centrifugation. A given amount of *t*-butanol, ammonium sulfate and crude slurry were added to a centrifuge tube. The mixture was stirred well to dissolve the salt completely. Centrifugation was performed to accelerate the phase-forming. After the formation of three phases, each phase was carefully separated, the volume of the lower phase was noted and the content of APS and protein in the lower phase was determined. Since the fact that majority of protein was extracted into the middle phase and barely any protein was extracted into the upper phase, the extraction of protein into the middle phase was calculated by subtracting the mass of protein in the lower phase from total mass of protein added into TPP.

*2.3 Analysis of APS and protein*

The methods for analyzing APS and protein were reported in our previous

95 studies [15]. The APS concentration was analyzed using the phenol-$H_2SO_4$ method
96 [31]. The absorbance was measured at the wavelength of 490 nm using an UV–Vis
97 spectrophotometer (UV-2100, Unico, USA). The calibration curve for analysis of
98 APS is $Y$=11.89$X$-0.0394 with $r$=0.9998 (n=5) using mannose as the standard, where
99 $Y$ is the absorbance and $X$ is the concentration of mannose in the range of 0.02–0.1
100 mg/mL. The protein concentration was determined via the Bradford method [32]
101 using BSA as standard. Samples were measured at 595 nm using spectrophotometry.
102 The calibration curve for analysis of protein is $Y$=5.2943$X$+0.0004 with $r$=0.9994
103 (n=6), where $Y$ is the absorbance, and $X$ is the concentration of BSA in the range of
104 0.02–0.12 mg/mL.

105 The extraction efficiency of APS ($E_a$) into the lower phase was defined in Eq.
106 (1):

107 $$E_a = \frac{C_a V_a}{m_a} \times 100\% \quad (1)$$

108 The extraction efficiency of protein ($E_p$) into the middle phase was defined in Eq.
109 (2):

110 $$E_p = (1 - \frac{C_p V_p}{m_p}) \times 100\% \quad (2)$$

111 $m_a$ and $m_p$ represent the amount of APS and protein in the crude slurry of aloe
112 pulp powder added. $C_a$ and $V_a$ represent the APS concentration and volume in the
113 lower phase. $C_p$ and $V_p$ represent the protein concentration and volume in the lower
114 phase.

115 *2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

116 SDS-PAGE of the samples was performed according to the method as reported

by Laemmli [33] with slight modification. Protein samples were loaded onto the electrophoresis gels, which were composed of 5% stacking and 12% separating gels. The electric current was 15 mA for the stacking gel and then 40 mA for the separating gel. After electrophoresis, the gel was stained for 2-3 h with staining solutions (0.05% (w/v) Coomassie brilliant blue R-250 in 45.4% (v/v) methanol, and 9.2% (v/v) acetic acid). The gel was then destained (7.5% (v/v) acetic acid and 5.0% methanol).

## 3. Results and Discussion

### *3.1 Optimization of the TPP*

Ammonium sulfate and *t*-butanol concentration, temperature, and pH were considered to be the critical parameters for the evaluation of TPP. Therefore, it was necessary to optimize the conditions for obtaining the maximum extraction efficiency.

Ammonium sulfate plays an important role in the TPP system, which flocculates the protein to the surface of the aqueous solution due to the salting-out effect. The influence of ammonium sulfate was investigated in the mass range of 0.8-2.8 g (mass fraction of 11.78%-33.37%). As shown in the Fig. 1(a), the extraction efficiency of protein increased with the increasing of salt, whereas no statistically significant increase (Student's *t*-test, $p>0.05$) was observed when the salt concentration was increased from 22.25% to 33.37%. The maximum extraction efficiency of APS was obtained at a 26.35% salt concentration, then it decreased with further increase of the salt concentration. This could be explained by the hypothesis that the stronger salting-out effect makes less free water available to dissolve the APS, and ammonium

sulfate flocculates the APS, leading to its separation into the middle phase. In light of these results, 26.35% (w/w) ammonium sulfate was chosen for further studies.

Compared with other alcohols, such as *n*-butanol, isobutanol and *n*-propanol, *t*-butanol has been widely considered to be the best solvent in TPP, which can increase the buoyancy of the precipitated protein by binding to it, resulting in its floatation above the denser aqueous salt layer [16, 34]. The influence of *t*-butanol was investigated in the volume range of 1.0-6.0 mL (mass fraction of 11.62%-44.09%). As shown in Fig. 1(b), higher extraction efficiencies of APS were obtained when a lower amount of *t*-butanol was added. The reason is that more *t*-butanol can make APS flocculate and enhance its buoyancy, leading to the partitioning of APS into the middle phase. Therefore, the extraction efficiency decreased with further increasing of *t*-butanol. The extraction efficiency of protein did not increase significantly (Student's *t*-test, $p>0.05$) with an increase of *t*-butanol concentration from 20.82% to 44.09%. More *t*-butanol can enhance the buoyancy of the precipitated protein making the protein more stable in the interface. Therefore, the 20.82% (w/w) *t*-butanol concentration was more suitable for simultaneous consideration of the extraction efficiency and cost.

Temperature also played an important role in TPP, and the effect of temperature was investigated in the range of 25-45 °C. The results in Fig. 1(c) showed that the lower temperature (below 35 °C) was more suitable for the extraction of APS and protein. The mass transfer velocity is raised, and more APS could dissolve into the aqueous phase with the increasing of temperature. However, a higher temperature will

cause consumption of more energy. The maximal extraction efficiency of protein was obtained at 30 ˚C as higher temperatures will accelerate the dissolution of the protein at the liquid-solid interface. In addition, the protein tends to be more stable at mild temperatures. Finally, the extraction can be processed by slight heating to attain a mild temperature of 30 ˚C. Both Thorat [35] and Gupta [36] reported the temperature of 20-40 ˚C to be a better range for carrying out TPP, but this was difficult to justify in view of complexity of the factors involved.

B-R (Britton-Robinson) buffer was used to adjust the pH of the aqueous solution to a range of 3.29-8.36. As shown in Fig. 1(d), the extraction efficiency of APS was not significantly increased (Student's *t*-test, $p>0.05$) from pH 3.29 to 6.37, and thus the weakly acidic circumstance was more suitable likely because there are some weakly acidic saccharides in aloe [12, 37]. Higher protein extraction efficiencies were obtained at the pH range of 5.5-7.5, indicating that the protein was more stable at this pH range, which is in accordance with our previous studies [15]. For example, one aloe protein (molecular mass of 11.8 kDa) reported by Siritapetawee has an isoelectric point (pI) of approximately 7.43 [8], and this protein is more stable near this pI. Therefore, it is necessary to adjust the system pH close to 6.5 for simultaneous consideration of the extraction of APS and proteins.

**Fig. 1**

*3.2 Recovery of t-butanol and removal of salt from APS*

*t*-butanol has a mild boiling point of 82.4 ˚C at 101.3 kPa, thus it can be easily evaporated and recycled. However, just as Przybycien reported [38], the use of

process scale quantities of *t*-butanol has restricted the industrial application of TPP as a chromatography alternative because the *t*-butanol has a similar flash point and volatility to that of ethanol. In future studies, other green and safe solvents (such as ionic liquid) can be used for substituting the volatile organic solvents in TPP.

To obtain APS with high purity, a dialysis was performed to remove the salt and some small molecular weight impurities using a dialysis membrane (D45 mm, MWCO 8000-14000). The average molecular mass of the APS was 1100 kDa, as Gu reported [7]. Other impurities, such as salt and small molecules, were easily removed by this dialysis membrane. The TPP was compared with two types of ATPS reported for extraction and purification of APS [14, 15]. The purity of APS was determined from the crude extract and after purification, and the results are shown in Table S1. TPP can isolate the APS and protein via one-step extraction; however, ATPS required a pretreatment method of alcohol precipitation to obtain crude APS with protein being discarded. The purity of APS increased from 28.4% in the crude slurry to 81.7% via TPP coupled with dialysis.

Furthermore, the UV spectrum of protein and the FT-IR spectrum of APS before and after TPP are shown in Figs. S2 and S3. The samples after TPP agreed well with the crude extract, demonstrating that the protein and APS show no structural change after TPP. TPP is a mild and effective method for the purification of aloe protein and APS.

*3.3 SDS-PAGE analysis of aloe protein*

The result of SDS-PAGE is shown in Fig. 2. The molecular weight of the aloe

protein was found to be approximately 10-15 kDa, which is consistent with the results reported by Siritapetawee [8] and Das [9]. Meanwhile, the band in Lane 2 is clearer than that in Lane 3, but the sample concentration of purified aloe protein was lower than that of crude slurry and the loaded sample volume is similar, in addition, the protein concentrations in TPP purified protein and crude slurry were determined via the Bradford method, indicating the concentration of purified protein was much larger than that of crude extract [39].

**Fig. 2**

## 4. Conclusion

TTP was used for simultaneous purification of APS and aloe protein via a single-step extraction. Under the optimized conditions of 26.35% (w/w) ammonium sulfate and 20.82% (w/w) *t*-butanol at 30 ˚C with pH 6.5, higher extraction efficiencies of APS and protein were obtained. APS in the lower phase was further purified by dialysis with a purity of 81.7%. The molecular weight of this obtained protein was approximately 10-15 kDa as determined via SDS-PAGE analysis.

## Acknowledgements

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## References

[1] Pugh N, Ross SA, ElSohly MA, Pasco DS. Characterization of aloeride, a new

high-molecular-weight polysaccharide from Aloe vera with potent immunostimulatory activity. J Agr Food Chem 2001;49:1030-1034.

[2] Aysan E, Bektas H, Ersoz F. A new approach to postoperative peritoneal adhesions: Prevention of peritoneal trauma by aloe vera gel. Eur J Obstet Gyn R B 2010;149:195-198.

[3] Anirban R, Gupta SD. A panoptic study of antioxidant potential of foliar gel at different harvesting regimens of Aloe vera L. Ind Crop Prod 2013;51:130-137.

[4] Atherton P. Aloe vera revisited: Review of Aloe gel. Brit J Phytother 1998;4:176-183.

[5] Ray A, Gupta SD, Ghosh S. Evaluation of anti-oxidative activity and UV absorption potential of the extracts of Aloe vera L. gel from different growth periods of plants. Ind Crop Prod 2013;49:712-719.

[6] Choi SW, Son BW, Son YS, Park YI, Lee SK, Chung MH. The wound-healing effect of a glycoprotein fraction isolated from aloe vera. Brit J Dermatol 2001;145:535-545.

[7] Gu W, Wang Y, Wu T, Li W, Wang H, Xia W. Linear sweep voltammetric studies on the complex of alizarin red s with aloe polysaccharide and determination of aloe polysaccharide. Carbohyd Res 2012;349:82-85.

[8] Siritapetawee J, Sojikul P, Soontaranon S, Limphirat W, Thammasirirak S. A protein from aloe vera that inhibits the cleavage of human fibrin(ogen) by plasmin. Appl Biochem Biotech 2013;170:2034-2045.

[9] Das S, Mishra B, Gill K, Ashraf MS, Singh AK, Sinha M, Sharma S, Xess I, Dalal

K, Singh TP, Dey S. Isolation and characterization of novel protein with anti-fungal and anti-inflammatory properties from Aloe vera leaf gel. Int J Biol Macromol 2011;48:38-43.

[10] Gowda DC, Neelisiddaiah, B., Anjaneyalu, Y. V. Structural studies of polysaccharides from aloe vera. Carbohyd Res 1979;72:201-205.

[11] t Hart LA, Berg AJJvd, Kuis L, Dijk Hv, Labadie RP. An anti-complementary polysaccharide with immunological adjuvant activity from the leaf parenchyma gel of Aloe vera. Planta Medica 1989;55:509-512.

[12] Zhu Y, Chen YX, Wu JH, Tan RX. Isolation, purification of acidic polysaccharide from Aloe vera and its anti- inflammatory activity. Chinese J Nat Med 2007;5:197-200.

[13] Chang XL, Xu H, Wang JJ, Wang WH, Feng YM. Research on water soluble polysaccharides separated from skin juice, gel juice and flower of Aloe ferox Miller. Food Sci Technol Res 2013;19:901-907.

[14] Xing JM, Li FF. Separation and purification of aloe polysaccharides by a combination of membrane ultrafiltration and aqueous two-phase extraction. Appl Biochem Biotech 2009;158:11-19.

[15] Tan ZJ, Li FF, Xu XL, Xing JM. Simultaneous extraction and purification of aloe polysaccharides and proteins using ionic liquid based aqueous two-phase system coupled with dialysis membrane. Desalination 2012;286:389-393.

[16] Dennison C, Lovrien R. Three phase partitioning: Concentration and purification of proteins. Protein Expres Purif 1997;11:149-161.

[17] Rather GM, Mukherjee J, Halling PJ, Gupta MN. Activation of alpha chymotrypsin by three phase partitioning is accompanied by aggregation. PloS One 2012;7:e49241.

[18] Narayan AV, Madhusudhan MC, Raghavarao KSMS. Extraction and purification of ipomoea peroxidase employing three-phase partitioning. Appl Biochem Biotech 2008;151:263-272.

[19] Chaiwut P, Pintathong P, Rawdkuen S. Extraction and three-phase partitioning behavior of proteases from papaya peels. Process Biochem 2011;45:1172-1175.

[20] Avhad DN, Niphadkar SS, Rathod VK. Ultrasound assisted three phase partitioning of a fibrinolytic enzyme. Ultrason Sonochem 2014;21:628-633.

[21] Gaur R, Sharma A, Khare SK, Gupta MN. A novel process for extraction of edible oils Enzyme assisted three phase partitioning (EATPP). Bioresource Technol 2007;98:696-699.

[22] Shah S, Sharma A, Gupta MN. Extraction of oil from Jatropha curcas L. seed kernels by combination of ultrasonication and aqueous enzymatic oil extraction. Bioresource Technol 2005;96:121-123.

[23] Wang HH, Chen CL, Jeng TL, Sung JM. Comparisons of alpha-amylase inhibitors from seeds of common bean mutants extracted through three phase partitioning. Food Chem 2011;128:1066-1071.

[24] Kurmudle NN, Bankar SB, Bajaj IB, Bule MV, Singhal RS. Enzyme-assisted three phase partitioning: A novel approach for extraction of turmeric oleoresin.

Process Biochem 2011;46:423-426.

[25] Roy I, Sharma A, Gupta MN. Three-phase partitioning for simultaneous renaturation and partial purification of Aspergillus niger xylanase. BBA-Proteins Proteom 2004;1698:107-110.

[26] Bayraktar H, Onal S. Concentration and purification of alpha-galactosidase from watermelon (Citrullus vulgaris) by three phase partitioning. Sep Purif Technol 2013;118:835-841.

[27] Li ZB, Jiang FF, Li Y, Zhang X, Tan TW. Simultaneously concentrating and pretreating of microalgae Chlorella spp. by three-phase partitioning. Bioresource Technol 2013;149:286-291.

[28] Vidhate GS, Singhal RS. Extraction of cocoa butter alternative from kokum (Garcinia indica) kernel by three phase partitioning. J Food Eng 2013;117:464-466.

[29] Roy I, Gupta MN. Three-phase affinity partitioning of proteins. Anal Biochem 2002;300:11-14.

[30] Dogan N, Tari C. Characterization of three-phase partitioned exo-polygalacturonase from Aspergillus sojae with unique properties. Biochem Eng J 2008;39:43-50.

[31] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-356.

[32] Bradford MM. A rapid and senstive method for the quantitation of microgram

quantities of protein utilizing the principle of protein-dye building. Anal Biochem 1976;76:248-254.

[33] Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680 - 685.

[34] Rajeeva S, Lele SS. Three-phase partitioning for concentration and purification of laccase produced by submerged cultures of Ganoderma sp. WR-1. Biochem Eng J 2011;54.

[35] Garg R, Thorat BN. Nattokinase purification by three phase partitioning and impact of t-butanol on freeze drying. Sep Purif Technol 2014;131:19-26.

[36] Sharma A, Gupta MN. Three phase partitioning as a large-scale separation method for purification of a wheat germ bifunctional protease/amylase inhibitor. Process Biochem 2001;37:193-196.

[37] Yan JC, Cui CY, Zhang Y, Chu RH. Separation, purification and structural analysis of polysaccharides from aloe. Chem J Chinese U 2003;24:1189-1192.

[38] Przybycien TM, Pujar NS, Steele LM. Alternative bioseparation operations: life beyond packed-bed chromatography. Curr Opin Biotech 2004;15:469-478.

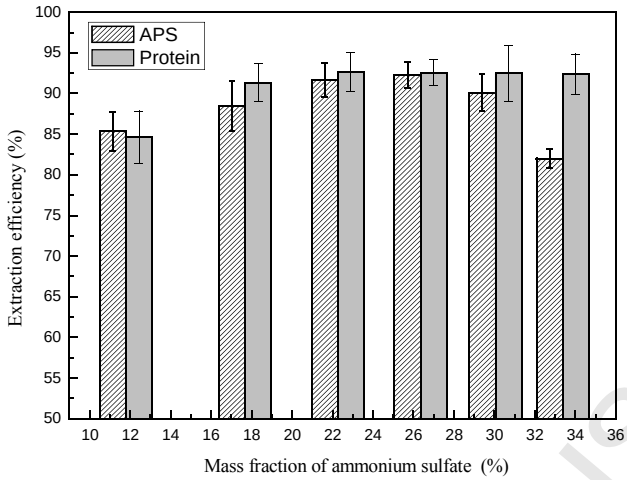
[39] Zhao L, Peng YL, Gao JM, Cai WM. Bioprocess intensification: an aqueous two-phase process for the purification of C-phycocyanin from dry Spirulina platensis. Eur Food Res Technol 2014;238:451-457.

**Tables and Figure captions:**

**Fig. 1** Effect of (a) ammonium sulfate concentration, (b) *t*-butanol concentration, (c) temperature, and (d) pH on extraction efficiencies of APS and protein.

**Fig. 2.** SDS-PAGE analysis of aloe protein [Lane 1: molecular weight markers (10–170 kDa), Lane 2: TPP purified aloe protein (The sample concentration is 0.02 g/mL), and Lane 3: crude slurry (The sample concentration is 0.06 g/mL)].

(a)

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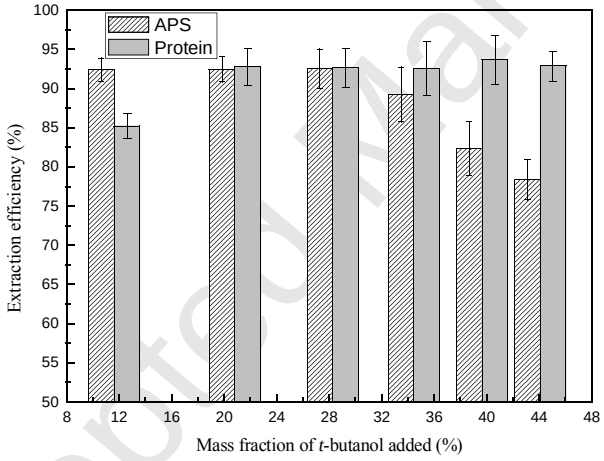
(b)

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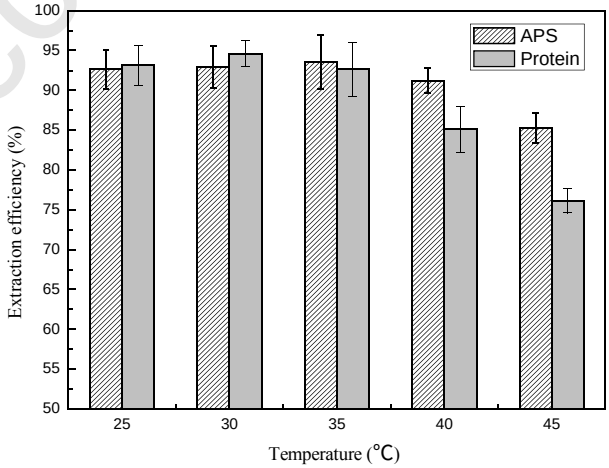
(c)

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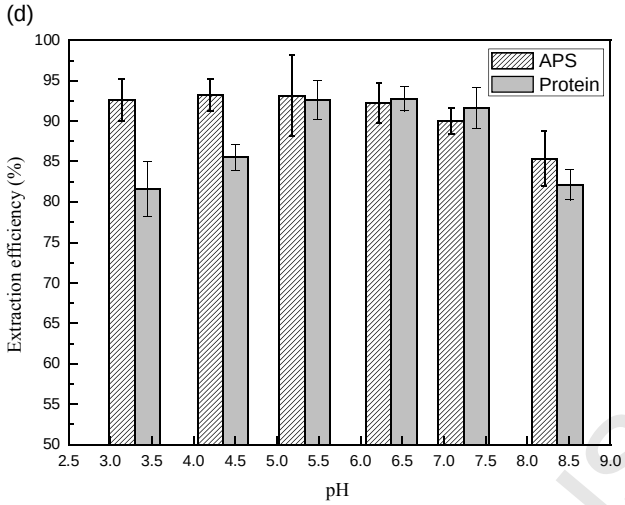


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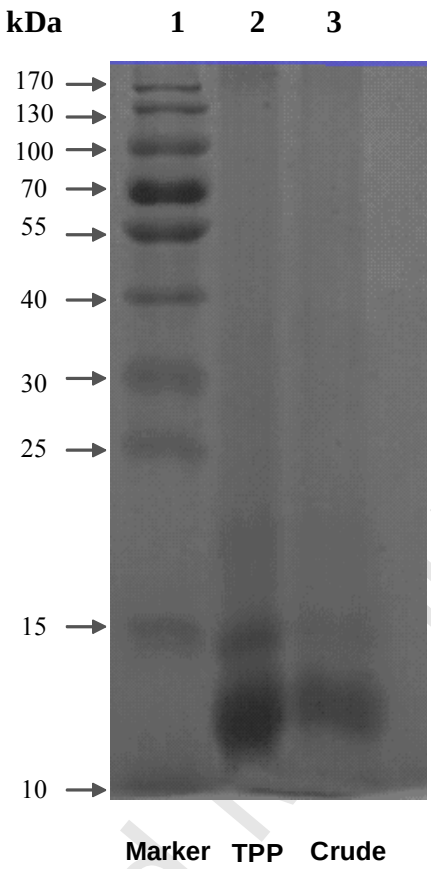


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